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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
A61K 37/64, C07K 13/00 // C12N 15/15	A1	WO 92/22320
		(43) International Publication Date: 23 December 1992 (23.12.92)

(21) International Application Number: PCT/US92/04452

(22) International Filing Date: 27 May 1992 (27.05.92)

(30) Priority data:

716,039	14 June 1991 (14.06.91)	US
859,781	30 March 1992 (30.03.92)	US

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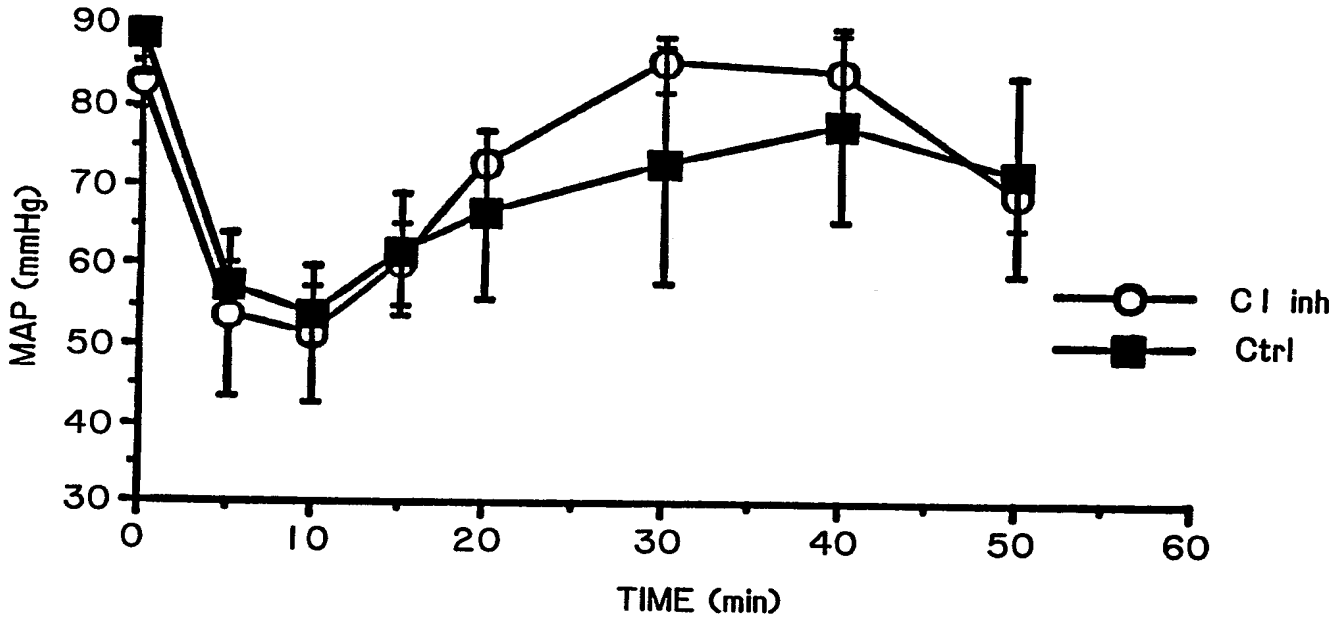
(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: C1 INHIBITOR VARIANTS AND TREATING INFLAMMATORY RESPONSE WITH C1 INHIBITOR



(57) Abstract

A method of treating a systemic inflammatory response in a mammal comprising administering to the mammal a therapeutically effective amount of C1-INH or C1-INH variant in an amount sufficient to treat the systemic inflammatory response. Among the acquired systemic inflammatory syndromes treated are hypotensive septic shock, adult respiratory distress syndrome, multiple organ system failure and preeclampsia.

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C1 INHIBITOR VARIANTS AND TREATING INFLAMMATORY RESPONSE WITH C1 INHIBITOR

FIELD OF THE INVENTION

This invention relates to the treatment of acquired systemic inflammatory responses such as septic shock, adult respiratory syndrome (ARDS), multiple organ system failure and preeclampsia. It also relates to improved forms of C1 inhibitor ("C1-INH").

BACKGROUND OF THE INVENTION

C1-INH is a plasma glycoprotein that belongs to the superfamily of serine protease inhibitors (serpins). C1-INH has been shown to inhibit activated components of the classical pathway of complement (C1r and C1s) and the intrinsic coagulation system (factor XIa, factor XIIa, and kallirein. C1-INH isolated from plasma has been used to treat hereditary angioedema where there is an inborn C1-INH deficiency having autosomal dominant characteristics. C1-INH deficiency is also associated with lymphoproliferative disorders such as idiopathic cutaneous necrotizing angiitis believed to be an immune-complex disease associated with hypocomplementemia (Harrison's Principles of Internal Medicine ,11th Edition, McGraw-Hill, Inc. New York,1987, p1411). C1-INH has not been tested in the therapeutic treatment of septic shock or adult respiratory distress syndrome (ARDS), multiple organ system failure or preeclampsia. The human C1-INH, a 105kDa glycoprotein, has been cloned and expressed in recombinant cell culture (Eldering et al *J. Biol. Chem.*, **263**:11776-11779, 1988).

Septic shock is the most common cause of death of humans in intensive care units in the United States (Parillo, J. E., *Ann. Rev. Med* **40**:469-485, 1989). It is usually initiated by a local nidus of infection that invades the blood stream. Incidences of shock can arise from sepsis (infections with either gram negative, gram positive bacterial or fungal microorganisms). All these organisms seem to induce a common pattern of cardiovascular dysfunction. In recent years aggressive fluid infusion therapy has been accepted as a primary means of treatment for septic shock. Adequate repletion of fluid is associated with an elevated cardiac output and low vascular resistance. Despite treatment, septic shock results in a severe decrease in systemic vascular resistance and generalized blood flow maldistribution. Aggressive therapy reverses shock and death in about 50% of the cases. Unresponsive hypotension resulting from a very low vascular resistance cannot be corrected by fluid infusion. Volume resuscitation is the first therapeutic step in the management of patients with septic shock. If hypotension persist, vasopressor agents are used. Dopamine is administered to raise the mean blood pressure to at least 60 mm Hg. If the does of dopamine exceeds 0.02 mg/kg per minute, the vasopressor, typically norepinephrene is used to maintain a mean blood pressure of 60 mm Hg (Parillo et al., *Annals of Internal Medicine*, **113**: 227-242, (1990). Among those subjects that die from septic shock, approximately 75% die from persistent hypotension and the remainder due to multiple organ system failure (Parillo, J. E.

supra). In septic shock, a decrease in vascular resistance with a subsequent increase in cardiac output is considered the initial hemodynamic abnormality. The reason for this decrease in vascular resistance is not known. Sepsis often leads to septic shock but the terms are not synonymous. WO 91/06650 teaches that the use of C1-INH (having certain mutations at positions 440 and 442) for the treatment of patients at risk of developing sepsis or that have developed sepsis. WO 91/06650 is silent about the use of the C1-INH mutants for sepsis that has progressed into the stage of shock.

The increase in cardiac output and vasodilation is attributed to the action of inflammatory mediators. The contact system of intrinsic coagulation (Hageman factor (factor XII)-dependent pathway) and the complement system are excessively activated in sepsis and septic shock, especially in cases of fatal septic shock. The Hageman factor system consists of factor XII, prekallikrein, and high molecular weight kininogen which participate in the generation of many vasoactive mediators such as bradykinin, coagulation factor XIIa, factor XIIf and C5a. Thus these mediators may play a role in the pathogensis of fatal shock. Bradykinin, factors XIIa, and XIIf are potent inducers of hypotension while C5a is an inducer of vasodilation and vasopermeability. The levels of factor XII, prekallikrein, and high molecular weight kininogen are decreased significantly during non-fatal shock but are most severely depressed during fatal septic shock to approximately 30%, 57% and 27% of normal values respectively. Such changes occur regardless of whether the septic state is caused by gram-positive or gram-negative bacterial agents.

Shock is defined as a state in which there is widespread, serious reduction of tissue perfusion, which, if prolonged, leads to generalized impairment of cellular function. Frequently shock is accompanied by hypotension and edema. In profound shock, a factor which impairs circulation is platelet aggregation and widespread disseminated intravascular coagulation in the bowel, kidney and other organs. Shock may lead to renal insufficiency, ARDS, multiple organ system failure, and death. Septic shock is a complex form of shock resulting from infection, especially gram-negative bacteremia with endotoxin release. According to Harrison's Principles of Internal Medicine (11th Edition, McGraw-Hill, Inc. New York,1987, p155) this form of shock is associated with fever, arteriovenous shunting, low systemic vascular resistance and arterial pressure, and an elevated cardiac output, with warm, dry skin, and in its late stages with vascular pooling, diminished venous return, reduced cardiac output, and hypotension despite increased vascular resistance, and with activation of complement leading to further cell damage.

Standard recommended therapy for septic shock is based upon the probable pathophysiologic mechanisms which can be summarized as follows: a) hypotension secondary to decreased cardiac volume; b) rapidly evolving processes causing hypotension and death within hours; and c) an infectious component and toxic processes caused by endotoxin and other mediators. Antibiotics, and abscess drainage are aimed at the microorganism, however, there is no specific therapy available to reverse the toxic process of sepsis. The

administration of fluids to maintain blood pressure and to maintain cardiac output are only partially effective. Effective treatment of septic shock, and related ARDS and multiple organ failure, is according to Harrison's *supra* not presently available.

Adult respiratory distress syndrome (ARDS) is a complex pulmonary disorder that affects hundreds of thousands annually, with a 50% mortality rate. (Carvalho et al J. Lab Clin Med, Vol 112, 270-277, Aug 1988). Although the exact pathogenesis of ARDS is not known, leukocytes, platelets, and the proteolytic pathways of coagulation and complement may mediate this illness. Sixty percent of patients with ARDS of various causes have simultaneous activation of the blood coagulation and fibrinolytic systems.

Multiple organ system failure is frequently associated with shock and with sepsis in need of surgical drainage and it involves multiple organs such as lungs (ARDS) kidney, liver, intestines, heart and brain. Most patients die despite maximal support of the respiratory and cardiovascular systems.

Preeclampsia is characterized by the appearance, during gestation or within 7 days of delivery, of a constellation of abnormalities which includes hypertension, edema and proteinuria. When hypertension is more severe, convulsions and coma may occur and is then termed eclampsia. Preeclampsia usually begins after the thirty-second week of pregnancy but may begin earlier, particularly in women with renal disease or hypertension. According to Harrison's Principles of Internal Medicine (11th Edition, McGraw-Hill, Inc. New York,1987, p1204) the pathogenesis of preeclampsia is not clearly understood. One hypothesis is that the uterus, like the kidney, synthesizes both vasoconstrictors (renin) and vasodialators (prostaglandins) and that an imbalance in their action is responsible for the severe hypertension. The formation of kallikrein is believed to be responsible for indirectly causing the sever hypertension. Kallikrein may cause high molecular weight kininogen to form bradykinin, thereby causing capillary permeability. Kallikrein can also elevate blood pressure by converting prorenin to renin (Derkx, F.H.M. et al., *J. Clin. Endocrinol. Metab.* **54**:343-348 (1982). Kallikrein is normally inhibited by C1-INH and consumption of C1-INH can lead to symptoms consistent with preeclampsia, namely edema. Reduced levels of C1-INH in normal pregnancy and significantly lower levels in patients with moderate preeclampsia has been reported (Halbmayer, et al., *Thrombosis and Haemostasis*, **65**:134-138 (1991)). Treatment of preeclampsia is bed rest and mild sedation and restriction of sodium intake. In more severe cases antihypertensive agents are employed.

C1-INH

C1-INH is a serine protease inhibitor involved in the regulation of several proteolytic systems in plasma including the complement, contact, coagulation and fibrinolytic systems. Davis et al., *Ann. Rev. Immunol.*, **6**:595-628 (1988). C1-INH is known to regulate those proteolytic systems by forming covalent complexes with specific serine protease components of each system. The amino acid sequence of human C1-INH and a DNA sequence encoding human C1-INH were described by Bock et al., *Biochemistry* **25**:4292-4301, 1986 and by Davis

et al.,*PNAS* **83**:3161-3165, 1986).

Several specific serine proteases whose activities are directly regulated by C1-INH have been identified. For instance, C1-INH is the only known inhibitor of C1r and C1s, both of which are activated fragments of the complement system component C1.

In addition, the art teaches that C1-INH is the most important inhibitor of the contact system because C1-INH is the primary inhibitor of both kallikrein and the activated forms of coagulation factor XII, including factor XIIa and factor XIIf (Hageman factor). Proud et al., *Ann. Rev. Immunol.*, **6**:49-83 (1988).

Deficiencies in C1-INH are known to cause serious and potentially life-threatening diseases. The best characterized example of a C1-INH deficiency is found in individuals with hereditary angioedema(HAE), also known as hereditary angioneurotic disorder, caused by a hereditary deficiency in the ability to produce C1-INH. Individuals with symptoms of HAE exhibit recurrent, acute, local circumscribed edema of the skin or mucosa, primarily on the extremities, face, larynx and gastrointestinal tract. Davis et al., *Ann. Rev. Immunol.*, **6**:595-628 (1988).

Another distinct type of C1-INH deficiency, termed acquired C1-INH deficiency, occurs in individuals who synthesize normal amounts of C1-INH, but cannot maintain sufficient concentrations of the inhibitor because of its increased catabolism. Individuals with acquired C1-INH deficiency exhibit the symptoms of hereditary angioedema. Whereas both hereditary angioedema and acquired C1-INH deficiency exhibit the typical laboratory profile of decreased levels of both C1-INH and complement systems components C4 and C2, the levels of complement system component C1q is decreased only in the acquired disease.

Presently, three distinct mechanisms have been proposed that create an acquired C1-INH deficiency. First, a C1-INH deficiency can be acquired when the amount of normally available C1-INH is consumed by an excessive amount of complement and/or contact system activation. Some of the activated components of each of the complement and contact systems can bind and inactivate C1-INH. In addition, activated contact system components can cleave C1-INH into inactive fragments. Zuraw et al., *J. Clin. Invest.*, **78**:567-575 (1986).

Second, a C1-INH deficiency can be acquired as the result of an anti-C1-INH autoimmune reaction. In such a case, C1-INH is synthesized, but anti-C1-INH autoantibodies bind the inhibitor and thereby prevent its ability to regulate serine protease activity. Third, a C1-INH deficiency can be acquired as a result of C1-INH being bound by complement-containing autoimmune complexes which are rapidly cleared from the circulation.

Presently, the specific physiological mechanism(s) controlling the blood concentration of C1-INH is not known. The primary site of C1-INH synthesis during the acute phase reaction to blood trauma has been proposed to be the liver. Davis et al., *Ann. Rev. Immunol.*, **6**:595-628 (1988); Johnson et al., *Science*, 173-553-554 (1971). In addition, the results of one *in vitro* study suggest that cultured hepatocytes synthesize C1-INH in response to interferon gamma. Zuraw et al., *Complement*, **4**:244, A319 (1987). In contrast, the source of C1-INH in

response to localized, non-traumatic inflammation appears to be cells of the monocyte/macrophase lineage. For instance, several *in vitro* studies have suggested that cultured monocytes can be stimulated to synthesize C1-INH by treatment with interferon gamma. See, Hamilton et al., *Biochem. J.*, **242**:809-815 (1987); Lotz et al., *J. Immunol.*, **79**:194, A280 (1987); Lotz et al., *J. Allerg. Clin. Immunol.*, **79**:194, A280 (1987); and Lappin et al., *Complement*, **4**:184, A160 (1987).

Methods for increasing C1-INH concentrations using interferon and/or interleukin-6 have been described in WO90/02570 (Lotz et al.) and a related publication Lotz et al. (*J. Immunology*, **139**:3382-3387, 1987). However, Lotz et al. disclose that α-interferon induces C1-INH, and that the uses for this C1-INH are strictly for complement inhibition. There is no recognition of the use of C1-INH for the treatment of septic shock, ARDS or multiple organ system failure, or preeclampsia.

Lotz et al. suggests that a method for increasing the intravascular level of C1-INH could be useful for treating disease conditions caused by C1-INH deficiencies. However, there have been no specific results demonstrating that septic shock pathology or ARDS is caused by a lack of C1-INH or that administration of C1-INH relieves septic shock, ARDS, multiple organ system failure or preeclampsia. Commonly, the conclusion was that C1-INH activity decrease seen in septic shock or ARDS was caused by the pathological condition, due to activation of the contact system of plasma; and that it was lower prekallikrein levels that were associated with higher mortality (Carvalho et al., *J. Clin. Med.*, **112**:270, 1988).

To date there has been no study of C1-INH effects on septic shock. This is not surprising in view of the fact that C1-INH is generally described as an agent inhibiting coagulation factors. U.S. Pat. No. 4,915,945 describes a method for the preparation of C1-INH for use when blood comes into contact with surfaces such a heart-lung machine where there is activation of the coagulation cascade. (Nuijens et al, *J. Clin Inves*, **84**:443-450, 1989) speculated that cleavage of C1-INH played a major role in the development of fatal complications during sepsis. However, again the focus was on the contact and complement system. Previous studies, as by Martinex-Brotons et al., *Thrombosis and Haemostasi*, **58**(2):709-713,1987, found that patients with septic shock, especially in fatal cases, showed a highly significant decrease in levels of factor XII, prekallikrein, high molecular weight, α_2-macroglobulin and antithrombin III. However, C1-INH activity was increased in uncomplicated sepsis and came back to normal or was slightly decreased in septic shock. The clear message being that C1-INH was essentially unchanged and that it was the activation of the kallikrein-kinin system that caused septic shock. This was consistent with findings made both earlier (Kalter et al., *J. Infectious Diseases*, **151**:1019-1027, 1985) and later (Nuijens et al., *Blood*, **72**:1841-1848, 1988).

In view of the pronounced fatal effects of septic shock, adult respiratory distress syndrome, and other systemic inflammatory clinical conditions such as multiple organ system failure and preeclampsia, it is an object of the present invention to provide a therapeutic

method comprising the administration of C1-INH in an amount sufficient to treat or prevent such clinical conditions. It an object of the present invention to reduce the general systemic inflammatory response that is the cause of septic shock and other related clinical conditions such as hypotension and associated edema. It is an additional object of the present invention to prepare amino acid sequence and other variants of C1-INH that do not substantially adversely affect the biological activity of the peptide but result in an increased ability to treat or prevent acquired systemic inflammatory responses. These and other objects of the invention will be apparent from the specification as a whole.

SUMMARY OF THE INVENTION

Described is a method of treating adult respiratory distress syndrome, multi-organ system failure or preeclampsia in a mammal comprising administering to the mammal a therapeutically effective amount of C1-INH. A preferred form of C1-INH is human C1-INH. C1-INH also is used for the treatment of septic shock, i.e. C1-INH unexpectaedly is effectively even when administered after the patient exhibits edema, hypotension and/or other threatening symptoms of septic shock as described above.

A pharmaceutical composition useful for reducing septic shock syndrome is a therapeutically effective amount of human C1-INH in a pharmaceutically acceptable carrier. This pharmaceutical composition may be used for reducing any acquired systemic inflammatory response such as septic shock, adult respiratory distress syndrome, multiple organ system failure and preeclampsia. The pharmaceutical composition may also be used to treat hypotension and edema. Also described are C1-INH variants useful in treating septic shock, adult respiratory distress syndrome, multiple organ system failure and preeclampsia. These variants generally include substitutions at one or more of residues A439-R444 inclusive.

DESCRIPTION OF THE FIGURES

Figure 1 illustrates the mean arterial pressure (MAP) in rats treated with or without a 1mg bolus injection of C1-INH administered three minutes after a 15mg/kg injection of LPS from *Salmonella Enteritidis.*

Figure 2 illustrates the mean arterial pressure (MAP) in rats treated with or without an infusion of 8mg of C1-INH (2mg/ml; 4ml over 1 hr.) started 3 min after a bolus injection (15mg/kg) of LPS from *Salmonella Enteritidis.*. The control (ctrl) rats received only a protein (Ovalbumin) infusion. There were 4 animals per group.

Figure 3 illustrates the mean arterial pressure (MAP) in rats receiving a low dose of C1-INH infused over 60 min. A solution of 1mg/ml was administered at 0.035ml/min for a total of 2mg/animal. The infusion started 3 min after an IV bolus injection of LPS from *Salmonella Enteritidis.*. There were 4 animals per group.

Figure 4 illustrates the mean arterial pressure (MAP) in rats sensitized with tumor

necrosis factor (TNF). The sensitized animals received LPS (15mg/kg) from *Salmonella Enteritidis.* followed 3 minutes later by a bolus injection of C1-INH (1mg/kg). There were 5 animals in each group.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention describes methods of using C1-INH to control septic shock, adult respiratory distress syndrome, multiple organ failure and preeclampsia. The therapeutic administration of C1-INH results in the modulation of such inflammatory responses and a reversal of clinical symptoms. In septic shock, C1-INH can block the triggering of factor XII, plasma kininogens and prekallikrein, thus reducing the activation of the contact system, the development of disseminated intravascular coagulation and hypotension. Hypotension (otherwise frequently leading to death) is reversed by administration of C1-INH. Administration of C1-INH or variants reverses hypotension induced by administered lipopolysaccaharide and C1-INH reduces or eliminates the shock normally produced.

The administration of C1-INH reduces or eliminates the pathophysiology of sepsis. This unexpected result is consistent only in part with previous observations. Plasma levels of C1-INH were known to increase during uncomplicated sepsis. However, reduction in the functional C1-INH and an increase in the inactivated C1-INH was observed in sepsis when sepsis was complicated with shock and adult respiratory distress syndrome. These higher levels of inactive C1-INH correlated with a higher frequency of mortality. It was undetermined whether such results were an effect or a cause of the mortality. In the present invention we have demonstrated that the administration of exogenous C1-INH following the onset of septic shock, or other systemic inflammatory disease syndromes, will replace the consumed C1-INH to ameliorate the development of fatal complications. Exogenously administered C1-INH reverses the development of hypotension by blocking the formation of vasoactive mediators, bradykinin, kallikrein, activated factor XIIa, factor XIIf and kallikrein and blocks the development of vascular permeability induced by C5a. The blocking of C5a and kallikrein formation further blocks the activation of human neutrophils which play a role in the development of microthrombi and multiple organ injury associated with sepsis.

C1-INH administered in the present invention may be any mammalian C1-INH, preferably human C1-INH as previously described. Variants of human C1-INH having superior clinical indications are suitable for therapeutic administration to reduce systemic inflammation reactions resulting in septic shock, adult respiratory distress syndrome, multiple organ failure and preeclampsia. C1-INH herein shall be construed to mean C1-INH amino acid sequence variants as well as the wild type protein.

C1-INH may be obtained from natural or recombinant sources. Example 4 illustrates the recombinant production of a human C1-INH variant. Once the entire coding sequence of DNA encoding such a variant C1-INH is attained (see WO 91/06650, Bock *et al., Biochemistry* **25**: 4292-4301 (1986) and Davis *et al., PNAS,* **83**: 3161-3165 (1986)), the complete sequence

containing an initiating methionine, presequence, and prosequence through the stop codon is inserted into a mammalian expression vector, such as pRK5, where under control of a promoter, such as the CMV promoter, C1-INH is expressed following transfection into a suitable host cell such as COS7 or 2935. Natural proteolytic processing to the mature soluble form will occur in the cell or in the cell-conditioned medium from where it may be purified. Alternatively, proteolytic enzymes may be added to the cell and/or to the conditioned medium to achieve the desired processing of the soluble C1-INH. Variant C1-INH may be produced using recombinant expression methods as described which incorporate DNA encoding C1-INH wherein the DNA contains specific deletions, insertions or substitutions of nucleotides as discussed below. DNA encoding the analogous C1-INH is obtained from other animal species via hybridization employing conventional methods. The preferred animals are mammals, particularly bovine, ovine, equine, feline, canine and rodentia, and more specifically rats, mice and rabbits.

Amino acid sequence variants of C1-INH are prepared by introducing appropriate nucleotide changes into C1-INH encoding DNA, or by *in vitro* synthesis of the desired C1-INH. Such variants include, for example, deletions from, or insertions or substitutions of, residues within C1-INH amino acid sequence. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Excluded from the scope of this invention are C1-INH variants or polypeptide sequences that are not novel and unobvious over the prior art. The amino acid changes also may alter post-translational processes of C1-INH, such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, and/or altering the intra-cellular location of C1-INH by inserting, deleting, or otherwise affecting the leader sequence of the native C1-INH.

In designing amino acid sequence variants of C1-INH, the location of the mutation site and the nature of the mutation will depend on C1-INH characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of other inhibitors adjacent to the located site.

A useful method for identification of certain residues or regions of C1-INH that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science*, **244**: 1081-1085, 1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of

the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the expressed C1-INH variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. These are variants from the published sequence, and may represent naturally occurring alleles (which will not require manipulation of C1-INH DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon C1-INH characteristic to be modified. Obviously, such variations that, for example, convert C1-INH into a known inhibitor, are not included within the scope of this invention, nor are any other C1-INH variants or polypeptide sequences that are not novel and unobvious over the prior art.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions may be introduced into regions of low homology with other inhibitors to modify the activity of C1-INH. Deletions from C1-INH in areas of substantial homology with any other inhibitors will be more likely to modify the biological activity of C1-INH more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of C1-INH in the affected domain, e.g., cysteine crosslinking, beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within C1-INH sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, and most preferably 1 to 3. Examples of terminal insertions include C1-INH with an N-terminal methionyl residue, an artifact of the direct expression of C1-INH in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of C1-INH molecule to facilitate the secretion of the mature C1-INH from recombinant host cells. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or lpp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of C1-INH include the fusion to the N- or C-terminus of C1-INH of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922, published 6 April 1989.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in C1-INH molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified

as the active site(s) of C1-INH, and sites where the amino acids found in C1-INH like inhibitors from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity.

Other sites of interest are those in which particular residues of C1-INH-like inhibitors obtained from various species are identical. These positions may be important for the biological activity of C1-INH. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in function or immunological identity of C1-INH are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

1) hydrophobic: norleucine, met, ala, val, leu, ile;
2) neutral hydrophilic: cys, ser, thr;
3) acidic: asp, glu;
4) basic: asn, gln, his, lys, arg;
5) residues that influence chain orientation: gly, pro; and
6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of C1-INH that are homologous with other inhibitors, or, more preferably, into the non-homologous regions of the molecule.

In one embodiment of the invention, it is desirable to inactivate one or more protease cleavage sites that are present in the molecule. These sites are identified by inspection of the encoded amino acid sequence. Where protease cleavage sites are identified, they are rendered inactive to proteolytic cleavage by substituting the targeted residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue.

In another embodiment, any methionyl residue other than the starting methionyl residue of the signal sequence, or any residue located within about three residues N- or C-terminal to each such methionyl residue, is substituted by another residue (preferably in accord with Table 1) or deleted. Alternatively, about 1-3 residues are inserted adjacent to such sites.

Any cysteine residues not involved in maintaining the proper conformation of C1-INH also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

Sites particularly suited for substitutions, deletions or insertions, or use as fragments, include, numbered from the N-terminus of C1-INH of Figure 5:

1) cysteines at C1-INH amino acid positions 101, 108, 183 and 406;
2) potential protease processing sites; and,
3) reactive centers such as arginine and threonine.

In some embodiments protease sensitive cleavage sites within C1-INH are modified or varied so as to become resistant to proteolytic attack, particularly by human neutrophil elastase. Optimally, the C1-INH variant is at least about 50% less sensitive to proteolytic cleavage than is wild-type C1-INH but the variant nonetheless is able to form an inhibiting complex with

C1s or kallikrein. Such variants are covalently modified C1-INH in which the changes are amino acid sequence variations (substitutions, insertions and/or deletions) or *in vitro* covalent substitutions of target residue side chains. For example, C1-INH proteolytic cleavage sites or relevant residues within the sites are substituted, deleted, residues are inserted adjacent to the site, or a polymeric substituent is inserted at or adjacent to a cleavage site. A glycosylation site is introduced sufficiently close to the cleavage site that the carbohydrate sterically hinders the access of the site to the protease. As an example, S14 or S41 are converted to O-linked glycosylation sites by introducing O-linked recognition sites in flanking residues by making appropriate substitutions. Similarily, N-linked recognition sites are introduced, e.g., substitution of A443R444T445 with N443R444T445.

Preferred mutations are protease resistant variants in the reactive center domain. This domain extends about from residues 438 to 449. For example residues 440 or 442 are substituted to $I440X_1$ and $V442X_2$, where X_1 and X_2 are an amino acid residue other than I or V, respectively. X_1 may be L or V. X_2 may be A, G, R, L or T. X_1 also preferably may be F, M, A, NorL, G or P. X_2 also may be P, I, S, D, E, N, Q, H, K, M, F, W or NorL. X_1 also is S, T, D, E, N, Q, H, K, R, Y or W. In addition, mutations are introduced into one or more residues located adjacent to residues I440 and V442, i.e. S438, A439, S441, A443, R444 (reactive center arginine) and T445 (reactive center threonine). Residues 440 and/or 442 may remain I and V, respectively, when mutations are introduced into S438, A439, S441, A443 or R444. T445, S438 and/or S441 generally are independently mutated to a positively or negatively charged residue or a hydrophobic residue. A439 or A443 are generally independently mutated to a positively or negatively charged residue, a neutral hydrophilic residue, or a hydrophobic residue. R444 is mutated to another positively charged residue, a negatively charged residue, a neutral hydrophilic residue or a hydrophobic residue. S438 or S441 generally are independently mutated to T, D, E, N, Q, H, K, R, M, F, Y, W, P, G, A, V, NorL, L or I. A439 or A443 generally are independently mutated to V, L, NorL, S, T, D, E, N, Q, H, K, R, M, F, Y, W, P or G. R444 generally is independently mutated to M, F, Y, W, P, G, A, V, L, NorL, I, S, T, D, E, N, Q, H or K. T445 generally is substituted with P, a hydrophobic residue, S, a positively or negatively charged residue or an aromatic residue. The reactive center R444T445 also is rendered protease resistant by insertion of a prolyl residue between R and T.

Also included within the scope of this invention are variations introduced into proteolytic cleavage sites other than the reactive center. Cleavage at these sites often precedes cleavage of the reactive center of C1-INH by neutrophil elastase. These sites include for example S14-L15, E36-P37 and V40-S41. In accordance with this invention, variation is introduced at these sites so as to prevent proteolytic cleavage by enzymes encountered *in vivo*. Most commonly, this is accomplished by substitution of the residues flanking the cleavage site with other residues that interfere with the recognition of the site by the relevant protease. For example, S14 or S41 typically are independently mutated to a hydrophobic

residue, T, an aromatic residue, a negatively charged residue or a positively charged residue; L15 or V40 generally are independently substituted with other hydrophobic residues, an uncharged hydrophilic residue, an aromatic residue, a negatively charged residue or a positively charged residue; E36 usually is substituted with a hydrophobic residue, an uncharged hydrophilic residue, D, an aromatic residue, or a positively or negatively charged residue. P37 generally is substituted by G, another hydrophobic residue, an uncharged hydrophilic residue, an aromatic residue, or a positively or negatively charged residue. Preferably, S14 or L15, E36, and V40 or S41 are substituted with A. P37 preferably is substituted with G.

Also useful is covalent modification of the sites with polyethylene glycol, block copolymers of polyoxyethylene and polyoxypropylene, or other conventional polymers heretofore covalently linked to proteins to extend the half-life or reduce the immunogenicity of thereof. The cross-linking agent, degree of substitution and reaction conditions are selected by a routine screen of conventional bifunctional agents, preferably a bank of reagents each one of which reacts with a different side chain. It may be desirable to substitute a residue having a reactive side chain, e.g., C or K, for a protease site residue so as to provide a convenient anchor for polymer substituents.

Typically, one protease resistant variation is introduced into each of the four protease sites described above, and preferably only in the active center domain. Otherwise the C1-INH generally contains the wild type sequence. This notwithstanding, however, it is within the scope of this invention for the C1-INH to encompass other sequence variations or covalent modifications, e.g. fusions to non-C1-INH polypeptides, fragments of C1-INH which retain C1-INH activity, covalent linkage to water insoluble matrices, and the like. See also WO 91/06650.

The various variants are readily screened for resistance to proteolytic degradation by labelling them with a detectable moiety, e.g., a fluorescent molecule or a radioisotope, incubating them with the protease or protease-containing body fluids, and determining whether cleavage has occured, e.g. by measuring the molecular weight of the labelled C1-INH variant by SDS-PAGE, by mass spectroscopic analysis, by N-terminal sequencing or the like.

DNA encoding amino acid sequence variants of C1-INH is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of C1-INH. These techniques may utilize C1-INH nucleic acid (DNA or RNA), or nucleic acid complementary to C1-INH nucleic acid.

The DNA encoding native or variant C1-INH is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and

3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of C1-INH encoding DNA that is inserted into the vector. The native proC1-INH encoding DNA encodes a signal sequence at the amino terminus (5' end of the DNA encoding C1-INH) of the polypeptide that is cleaved during post-translational processing of the polypeptide to form the mature C1-INH. Native C1-INH is not, however, secreted from the cell as it contains a transmembrane domain and a cytoplasmic region in the carboxyl terminal region of the polypeptide. Thus, to form a secreted version of C1-INH the carboxyl terminal domain of the molecule, including the transmembrane domain, is ordinarily deleted. This truncated variant C1-INH may be secreted from the cell, provided that the DNA encoding the truncated variant retains the amino terminal signal sequence.

The C1-INH of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-and/or C-terminis of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of C1-INH encoding DNA that is inserted into the vector. Included within the scope of this invention are C1-INH with the native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native C1-INH signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native C1-INH signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ

plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of C1-INH DNA. However, the recovery of genomic DNA encoding C1-INH is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise C1-INH encoding DNA.

(iii) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli.*

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al., J. Molec. Appl. Genet.,* **1**: 327,1982), mycophenolic acid (Mulligan *et al., Science,* **209**: 1422,1980) or hygromycin (Sugden *et al., Mol. Cell. Biol.,* **5**: 410-413,1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up C1-INH nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the

transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes C1-INH. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of C1-INH are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, **77**: 4216, 1980. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding C1-INH. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding C1-INH, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418 (see U.S. Pat. No. 4,965,199).

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, **282**: 39, 1979; Kingsman *et al.*, *Gene*, **7**: 141, 1979; or Tschemper *et al.*, *Gene*, **10**: 157, 1980). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, **85**: 12, 1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to C1-INH nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as C1-INH to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in

culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding C1-INH by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native C1-INH promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of C1-INH encoding DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed C1-INH as compared to the native C1-INH promoter.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang *et al.*, *Nature*, **275**: 615, 1978; and Goeddel *et al.*, *Nature*, **281**: 544, 1979), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, **8**: 4057, 1980 and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, *Proc. Natl. Acad. Sci. USA*, **80**: 21-25, 1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding C1-INH (Siebenlist *et al.*, *Cell*, **20**: 269, 1980) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding C1-INH.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.*, **255**: 2073, 1980) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.*, **7**: 149, 1968; and Holland, *Biochemistry*, **17**: 4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT (Seq. ID #1) region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence (Seq. #2) that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of

these sequences are suitably inserted into mammalian expression vectors.

C1-INH transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504, published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with C1-INH sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication (Fiers *et al.*, *Nature*, **273**:113 (1978); Mulligan and Berg, *Science*, **209**: 1422-1427 (1980); Pavlakis *et al.*, *Proc. Natl. Acad. Sci. USA*, **78**: 7398-7402 (1981)). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment (Greenaway *et al.*, *Gene*, **18**: 355-360 (1982)). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Gray *et al.*, *Nature*, **295**: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature*, **297**: 598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, *Proc. Natl. Acad. Sci. USA*, **79**: 5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, **79**: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding C1-INH of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins *et al.*, *Proc. Natl. Acad. Sci. USA*, **78**: 993, 1981) and 3' (Lusky *et al.*, *Mol. Cell Bio.*, **3**: 1108, 1983) to the transcription unit, within an intron (Banerji *et al.*, *Cell*, **33**: 729, 1983) as well as within the coding sequence itself (Osborne *et al.*, *Mol. Cell Bio.*, **4**: 1293, 1984). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers (see also Yaniv, *Nature*, **297**:

17-18 (1982)) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to C1-INH encoding DNA, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding C1-INH. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, *Nucleic Acids Res.*, **9**: 309 (1981) or by the method of Maxam *et al.*, *Methods in Enzymology*, **65**: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding C1-INH. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of C1-INH that have C1-INH-like activity. Such a transient expression system is described in U.S. Pat. No. 5,024,939 issued June 18, 1991.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of C1-INH in recombinant vertebrate cell culture are described in Gething *et al.*, *Nature*, **293**: 620-625, 1981; Mantei *et al.*, *Nature*, **281**: 40-46, 1979; Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful expression plasmid for mammalian cell culture expression of C1-INH is pRK5 (EP pub. no. 307,247).

SELECTION AND TRANSFORMATION OF HOST CELLS

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* $_{x}$1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for C1-INH-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, **290**: 140 (1981); EP 139,383, published May 2, 1985), *Kluyveromyces* hosts (Pat. No. 4,943,529) such as, e.g., *K. lactis* (Louvencourt *et al.*, *J. Bacteriol.*, **737** (1983); *K. fragilis, K. bulgaricus, K. thermotolerans*, and *K. marxianus, yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070), Sreekrishna *et al.*, *J. Basic Microbiol.*, **28**: 265-278 (1988); *Candida, Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case *et al.*, *Proc. Natl. Acad. Sci. USA*, **76**: 5259-5263 (1979), and filamentous fungi such as, e.g, *Neurospora, Penicillium, Tolypocladium* (WO 91/00357, published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, *Biochem. Biophys. Res. Commun.*, **112**: 284-289 (1983); Tilburn *et al.*, *Gene*, **26**: 205-221 (1983); Yelton *et al.*, *Proc. Natl. Acad. Sci. USA*, **81**: 1470-1474 (1984) and *A. niger* (Kelly and Hynes, *EMBO J.*, **4**: 475-479 (1985)).

Suitable host cells for the expression of glycosylated C1-INH are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified (see, e.g., Luckow *et al.*, *Bio/Technology*, **6**: 47-55 (1988); Miller *et al.*, in *Genetic Engineering*, Setlow, J.K. *et al.*, eds., **Vol. 8** (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, *Nature*, **315**: 592-594 (1985)). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts.

Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain C1-INH DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding C1-INH is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express C1-INH DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences (Depicker *et al.*, *J. Mol. Appl. Gen.*, **1**: 561 (1982)). In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue (see EP 321,196, published 21 June 1989).

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture,* Academic Press, Kruse and Patterson, editors (1973)). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.*, **36**: 59, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* **77**: 4216 [1980]); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, **23**: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.*, **383**: 44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al., supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall

barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, *Gene*, **23**:315 (1983) and WO 89/05859, published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook *et al, supra,* is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216, issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, *J. Bact.*, **130**: 946 (1977) and Hsiao *et al.*, *Proc. Natl. Acad. Sci. (USA)*, **76**: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

CULTURING THE HOST CELLS

Prokaryotic cells used to produce C1-INH of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra.*

The mammalian host cells used to produce C1-INH of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.*, **58**:44 (1979), Barnes and Sato, *Anal. Biochem.*, **102**: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S. Pat. No. 5,122,469 filed on 3 October 1990, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

It is further envisioned that C1-INH of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding C1-INH currently in use in the field. For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired C1-INH. The control element does not encode C1-INH of this invention, but the DNA is present in the host cell

genome. One next screens for cells making C1-INH of this invention, or increased or decreased levels of expression, as desired.

DETECTING GENE AMPLIFICATION/EXPRESSION

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA,* **77**: 5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled where the labels are usually visually detectable such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, *Am. J. Clin. Path.*, **7**: 734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native C1-INH or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

PURIFICATION OF C1-INH AND VARIANT POLYPEPTIDE

The C1-INH may be recovered from a cellular fraction or supernatant or it may be recovered from the culture medium as a soluble polypeptide. A C1-INH may also be recovered as described in example 4.

C1-INH may be purified from fresh human plasma by the method of Pilatte, Hammer, Frank and Fries, (*Journal of Immunological Methods*, **120**: 37-43, 1989). Alternatively, C1-INH may be produced by recombinant methods as discussed above. The C1-INH then isolated from the recombinant culture by conventional means. One such method involves PEG fractionation, jacalin-agarose chromatography, and hydrophobic interaction chromatography

on phenyl-Sepharose. The amino acid sequence of human C1-INH and the DNA sequence encoding human C1-INH were described by Bock et al. (Biochemistry 25: 4292-4301 , 1986) and by Davis et al. (PNAS 83: 31613165, 1986). The activity of the isolated C1-INH protein may be assayed by monitoring inhibition of complement mediated lysis of antibody sensitized sheep red blood cells, by C1-INH binding to C1s and C1r (Quidel) and by inhibition of plasma kallikrein mediated breakdown of the synthetic substrate H-D-Prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride.

When C1-INH is expressed in a recombinant cell other than one of human origin, C1-INH is completely free of proteins or polypeptides of human origin. However, it is necessary to purify C1-INH from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to C1-INH. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The C1-INH may then be purified from both the soluble protein fraction (requiring the presence of a protease) and from the membrane fraction of the culture lysate, depending on whether C1-INH or variant is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica, heparin sepharose or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, Sephadex G-75.

C1-INH variants in which residues have been deleted, inserted or substituted are recovered in the same fashion as the native C1-INH, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a C1-INH fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-C1-INH column can be employed to absorb C1-INH variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenylmethylsulfonylfluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native C1-INH may require modification to account for changes in the character of C1-INH or its variants upon expression in recombinant cell culture.

COVALENT MODIFICATIONS OF C1-INH

Covalent modifications of C1-INH are included within the scope of this invention. Both native C1-INH and amino acid sequence variants of C1-INH may be covalently modified. One type of covalent modification included within the scope of this invention is a C1-INH fragment. C1-INH fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length C1-INH or C1-INH variant polypeptide. Other types of covalent modifications of C1-INH or fragments

thereof are introduced into the molecule by reacting targeted amino acid residues of C1-INH or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking C1-INH to a water-insoluble support matrix or surface for use in the method for purifying anti-C1-INH antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-

azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of modification of C1-INH is the formation of fusion proteins with a heterologous polypeptide. The heterologous polypeptide may be an anchor sequence such as that found in the decay accelerating system (DAF). The heterologous polypeptide may be a toxin such as ricin, pseudomonas exotoxin, gelonin, or other polypeptide that will result in target cell death. Still other proteins may be fused to C1-INH such as enzymes that result in cell death or inhibition such as nucleases, including both DNAse and RNAse. These heterologous polypeptides may alternatively be covalently coupled to C1-INH. Similarly, other molecules toxic or inhibitory to a target mammalian cell may be coupled to C1-INH, such as antisense DNA that blocks gene function or expression, and tricothecenes.

Another type of covalent modification of C1-INH included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in native C1-INH, and/or adding one or more glycosylation sites that are not present in the native C1-INH.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to C1-INH is conveniently accomplished by altering the

amino acid sequence such that it contains one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native C1-INH sequence (for O-linked glycosylation sites). For ease, C1-INH amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding C1-INH at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of C1-INH".

Another means of increasing the number of carbohydrate moieties on C1-INH is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- and O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330, published 11 September 1987, and in Aplin and Wriston (CRC Crit. Rev. Biochem., pp. 259-306 [1981]).

Removal of carbohydrate moieties present on the native C1-INH may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin *et al., Arch. Biochem. Biophys.*, **259**:52 (1987) and by Edge *et al.*, *Anal. Biochem.*, **118**:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo- glycosidases as described by Thotakura *et al.* (*Meth. Enzymol.*, **138**:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al.* (*J. Biol. Chem.*, **257**:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of C1-INH comprises linking C1-INH to various nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

One preferred way to improve the *in vivo* circulating half-life of C1-INH is to conjugate it to a polymer that confers extended half-life, such as conjugating polyethylene glycol (PEG) to C1-INH, was found to be an excellent way to increase the half-life. PEG is an non-immunogenic, linear, uncharged polymer with three water molecules per ethylene oxide unit which therefore can alter the hydrodynamic properties of the conjugated molecules

dramatically. (Maxfield, *et al, Polymer*, 16:505-509 (1975); Bailey, F. E., *et al*, in Nonionic Surfactants [Schick, M. J., ed] pp.794-821, 1967). Several enzymes for therapeutic usage were PEGylated to increase the *in vivo* half-life effectively (Abuchowski, A. *et al, J. Biol. Chem.* **252::**3582-3586, 1977; Abuchowski, A. *et al, Cancer Biochem. Biophys.*, **7**:175-186, 1984). PEGylation of IL-2(interleukin-2) was also reported to increase circulatory life as well as its potency (Katre, N.V. *et al, Proc. Natl. Acad. Sci.*, **84**:1487-1491 (1987); Goodson, R. *et al*., *Bio/Technology*, **8**:343-346, 1990). PEGylation of other molecules were reported to have reduced immunogenicity and toxicity (Abuchowski, A. *et al, J. Biol. Chem.*, **252**:3578-3581, 1977).

The C1-INH also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980).

C1-INH preparations are also useful in generating antibodies, as standards in assays for C1-INH (e.g., by labeling C1-INH for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant C1-INH, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of C1-INH molecule, such as affinity for a given antibody, is measured by a competitive-type immunoassay. The variant is assayed for binding affinity to complement factors or to other Ci-INH target proteins. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for native C1-INH in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

THERAPEUTIC COMPOSITIONS AND ADMINISTRATION OF C1-INH

Therapeutic formulations of C1-INH are prepared for storage by mixing C1-INH having the desired degree of purity (typically greater than 95%, and usually greater than 99% pure by weight of protein) with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, *supra*), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and

other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

C1-INH to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. C1-INH ordinarily will be stored in lyophilized form or in solution.

Therapeutic C1-INH compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

C1-INH used in the treatment of septic shock may be optionally combined with or administered in concert with other agents known for use in the treatment of septic shock, adult respiratory distress syndrome, pre-eclampsia or edema. Among the preferred agents are antibody specific for the CD18 or CD11a, 11b or 11c cell surface antigens.

The route of C1-INH administration is in accord with known methods, e.g., injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems as noted below. C1-INH is administered continuously by infusion or by bolus injection.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res.*, **15**:167-277 [1981] and Langer, *Chem. Tech.*, **12**:98-105 [1982] or poly(vinylalcohol)], polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *Biopolymers*, **22**:547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988). While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange,

stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release C1-INH compositions also include liposomally entrapped C1-INH. Liposomes containing C1-INH are prepared by methods known *per se*: DE 3,218,121; Epstein *et al., Proc. Natl. Acad. Sci. USA,* **82:** 3688-3692 (1985); Hwang *et al., Proc. Natl. Acad. Sci. USA,* **77:**4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamelar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal C1-INH therapy. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Another use of the present invention comprises incorporating C1-INH into formed articles. Such articles can be used in modulating or preventing the occurrence of shock.

An effective amount of C1-INH to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 0.1mg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. A preferred dosage is about 250 mg for the average adult patient. Typically, the clinician will administer C1-INH until a dosage is reached that achieves the desired effect. One desired dosage is that necessary to achieve a plasma concentration of about 200 µg/ml of plasma. The progress of this therapy is easily monitored by conventional assays. Ordinarily, C1-INH is administered by i.v. injection or infusion (e.g., for from 1 min to 6 hours).

IV administration of C1-INH reverses hypotension resulting from the IV administration of murine TNF alpha and endotoxin in the rat. This is illustrated in Example 2 where a model was established using an LPS challenge. All animals developed hypotension following IV administration with LPS. The C1-INH treated animals showed near normal blood pressures between 20-50 minutes as compared to control animals (Figure 4). This indicates that hypotension previously considered to lead to death now may be reversed by administering a sufficient dose of C1-INH.

The methods and procedures described herein with human C1-INH may be applied similarly to C1-INH from other mammals and to inhibitor variants. All references cited in this specification are expressly incorporated by reference. The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

ISOLATION OF C1-INH

C1-INH was purified from fresh human plasma by the method of Pilatte, Hammer, Frank and Fries (Journal of Immunological Methods 120; 37-43, 1989). The method involves PEG fractionation, jacalin-agarose chromatography, and hydrophobic interaction chromatography on phenyl-Sepharose. Fresh frozen human plasma was obtained and no exogenous protease inhibitors were added prior to homogenization. The plasma was clarified by centrifugation at 4000 rpm in a Beckman GSA rotor. Plasma proteins were precipitated with 21.4% PEG (PEG 3350) and the pellet discarded. The supernatant fluid was retreated with 45% PEG and the precipitate discarded following centrifugation for 30 minutes at 4000 rpm in a Beckman GSA rotor. The pellet was solubilized in 175 mM Tris pH 7.5 and applied to Jacalin Agarose column chromatography and eluted with 125 mM Melibose. The eluant which was adjusted to 400 mM $(NH_4)_2SO_4$ was applied to Phenyl Sepharose. The effluent was retained and dialyzed against phosphate buffered saline. The material was concentrated to 1 mg/ml. This plasma isolated C1-INH was used in all other experiments.

EXAMPLE 2

C1-INH REVERSES HYPOTENSION INDUCED BY THE ADMINISTRATION OF ENDOTOXIN

The ability of C1-INH to reverse hypotension induced by the therapeutic administration of endotoxin was evaluated in two experiments. In the first experiment, Wistar rats (250- 300 g) were anesthetized with phenobarbital sodium (130 mg/kg i.v.). The carotid artery was cannulated for blood pressure recording. All drugs were administered via a jugular vein cannula. Lipopolysaccharide(LPS) from Salmonella enteritidis (15 mg/Kg) was injected intravenously at t=0 min. This LPS dose was followed by a bolus dose of 1 mg C1-INH at t=3 min. Groups consisted of 4 treated and 4 control protein treated animals. A small and transient increase in blood pressure back to normotensive was observed in C1-INH treated animals as compared to control animals (Figure 1).

In a second experiment, a higher concentration of C1-INH was injected. The experimental animal was injected intravenously with a bolus of lipopolysaccharide from Salmonella enteritidis (15 mg/Kg). C1-INH was injected as an infusion of 8 mg of C1-INH (2 mg/ml at 4 ml over one hour) starting 3 minutes after the endotoxin. The control animal received an equivalent volume and amount of ovalbumin in 15mg/Kg. The mean arterial pressure was recorded. The administration of human plasma derived C1-INH induced an increase in mean arterial pressure in the rat following administration of endotoxin (Figure 2). The reversal of hypotension by C1-INH is dose dependent. A lower dose of C1-INH at 2 mg (0.5mg/ml x 4ml) over one hour infusion in rats did not result in the same reversal of hypotension (Figure 3).

EXAMPLE 3

C1-INH REVERSES HYPOTENSION FOLLOWING THE ADMINISTRATION OF TNF ALPHA AND ENDOTOXIN

In the rat the administration of C1-INH reverses the hypotension caused by the administration of murine TNF-α and endotoxin. To establish a model were the LPS challenge induces a more consistent outcome, Wistar rats were injected with TNF (0.7 mg/Kg) at t=-30 min. prior to LPS. Animals were injected intravenously with LPS (Salmonella enteritidis) at t=0 min. Five animals were treated with C1-INH (1 mg/Kg) at t=3 min. after LPS. Five control animals were treated with equivalent volume of saline. All animals developed hypotension following administration with LPS. C1-INH treated animals showed near normal blood pressures between 20-50 minutes as compared to control animals (Figure 4).

Intravenous administration of endotroin results in a drop in mean arterial pressure in rats, similar to humans with septic shock, In rats, endotoxin induces a decrease in plasma prekallikrein and kininogen levels similar to that in man. These plasma proteins likely play a role in the pathophysiology of septic shock. (Hogstrom, et al., *Acta Chir. Scand.*, **153**:161-164 (1987) and Aasin, et al., *Arch. Surg.*, **118**: 343-345). In humans, serial cardiovascular measurements and near-death cardiovascular determinations made to determine the mechanism of patient demise demonstrated that 76% died of persistent hypotension attributed to myocardial depression (14%) or a low systemic vascular resistance (62%). 24% died from multiple organ system failure. Thus a large majority of septic shock patients died of persistent hypotension (Parillo, *Ann. Rev. Med.* **40**:469-485).

Persistent hypotension is characteristic of end-stage septic shock and is believed to be the most refractory state of the disorder due to its advanced condition. Thus, it was extremely surprising that C1-INH was capable of salvaging the animals when treated at this stage of septic shock.

EXAMPLE 4

CLONING AND EXPRESSION OF HUMAN C1-INH

A human C1-INH was cloned and expressed in cultured chinese hamster ovary cells. The cDNA encoding human C1-INH was cloned out of two phage lambda libraries, one an oligo-dT primed library made from RNA from human fetal liver (L027), and the other a random primed library made from RNA from human adult liver (L015). Three probes were constructed based upon the published sequence of Davis *et al., PNAS*, **83**:3161-3165 (1986). One probe was specific for each third of C1-INH encoding DNA sequence, defined as the 5', the middle and 3' portions. The probes were end-labeled by gamma ^{32}P ATP with polynucleotide kinase and used to screen the libraries using 30% formamide for hybridization and in 0.4XSSC and 0.1% SDS for the washing step. The first screen utilized the middle oligonucleotide probe and the L027 library and 22 clones were isolated, however none extended all the way to the 5' end of C1-INH encoding DNA.

From the L015 library, 23 possible positives were picked and tested with the 5' and 3' probes. Five positive clones were detected with the 3' probe, and one clone with the 5' probe. This 5' clone and 3' clones were recombined by the unique middle Kpn 1 restriction site to form a large cDNA encoding the human C1-INH. This was inserted into a mammalian expression vector and tested for expression. There was no expression of C1-INH due to a deletion at the 5' end of C1-INH encoding DNA. Probes for the 5' end were used in a polymerase chain reaction to screen library L015 and 22 5' positive clones were detected and rescreened. One of these was identified and subcloned to repair the 5' end resulting in a complete DNA sequence encoding the human C1-inhibitor. This DNA sequence contains two novel nucleotide substitutions, G to A substitution at nucleotide #954 resulting in a change from arginine to lysine at C1-INH amino acid #306; and T to C substitution at nucleotide #1219 which does not result in a change in the amino acid threonine at position 395.

The complete C1-INH encoding DNA was inserted into a mammalian expression vector. This vector was introduced into CHO cells by the calcium phosphate coprecipitation method ((Graham et al. Virology **52**, 456-467, 1982). Half-confluent plates of cells (60mm) were exposed to 5 μg of plasmid DNA in 1 ml of precipitate for 6-8 hr. After a 15% (vol/vol) glycerol shock (Frost et al Virology **91**: 39-50, 1978), the cells were refed with culture medium. Two days later, the medium was aspirated, the cells washed once with phosphate buffered saline, and refed with 3 ml methionine and serum-free labeling medium containing 200 μCi of ^{35}S-Methionine (Amersham, 1000 Ci/mmol)). After overnight incubation, the labeling medium was collected, cleared of floating cells by a 1 min microfuge spin and 40 μl of protein A sepharose was added. The immunoprecipitation used commercially available antibody specific for C1-INH and the procedure was carried out as described by Hudziak et al (PNAS **84**:7159-7163, 1987).

The labeled proteins were separated on a 10% SDS polyacrylamide gel and analyzed by autoradiography. A band of protein with a molecular weight of 105-110 kDa was seen in cells transfected with C1-INH construct but not in cells transfected with plasmid alone. These results indicated that C1-INH had been synthesized using the DNA of encoding C1-INH based upon both molecular weight and immune specificity.

Claims:

1. A method of treating a systemic mammalian inflammatory response selected from the group consisting of adult respiratory distress syndrome, hypotensive septic shock, multiple organ system failure and pre-eclampsia, comprising administering to the mammal a therapeutically effective amount of C1-INH.

2. The method of claim 1 wherein said mammal is a human.

3. The method of claim 2 wherein said C1-INH is human C1-INH.

4. The method of claim 1 wherein said systemic inflammatory response is hypotensive septic shock.

5. The method of claim 1 wherein said systemic inflammatory response is adult respiratory distress syndrome.

6. The method of claim 1 wherein said systemic inflammatory response is multiple organ system failure.

7. The method of claim 1 wherein said systemic inflammatory response is preeclampsia.

8. A C1-INH variant which is resistant to proteolytic cleavage, provided, however, that the variant contains I440 and V442.

9. A C1-INH variant which is resistant to proteolytic cleavage, provided, however, that the variant does not include A440, G440, L440, T440, L442 or V442.

10. The variant of claim 9 which is resistant to neutrophil proteases.

11. The variant of claim 11 wherein the protease is human neutrophil elastase.

12. The variant of claim 11 wherein the variant is able to form an inhibitory complex with C1s or kallikrein.

13. The variant of claim 11 wherein the variant is about 50% less sensitive to proteolytic inactivation than is wild type C1-INH.

14. The variant of claim 9 wherein the variant comprises an amino acid sequence change at the reactive center arginine or threonine residues.

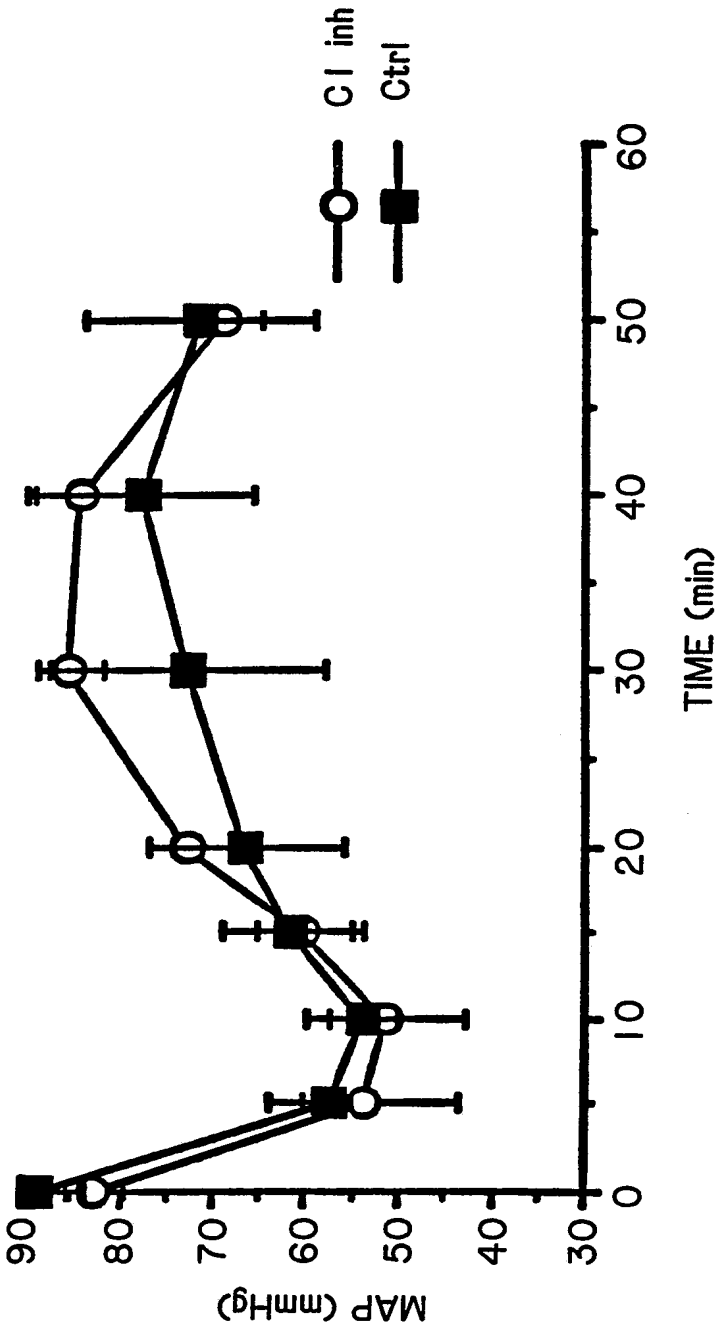
C I inh
Ctrl
MAP (mmHg)
90
80
70
60
50
40
30
0
10
20
30
40
50
60
TIME (min)

FIG. 1

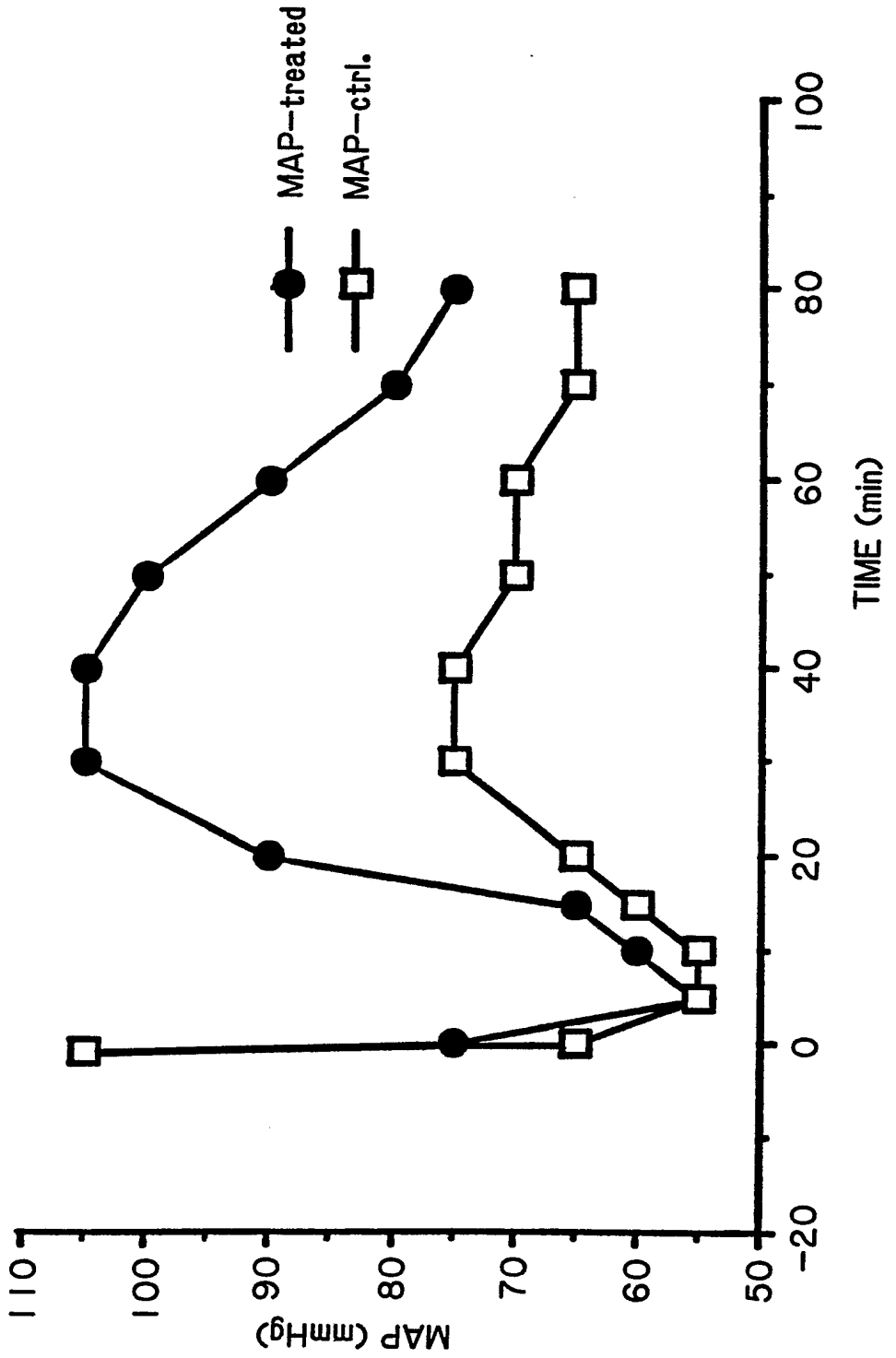
MAP-treated
MAP-ctrl.
MAP (mmHg)
110
100
90
80
70
60
50
-20
0
20
40
60
80
100
TIME (min)

FIG. 2

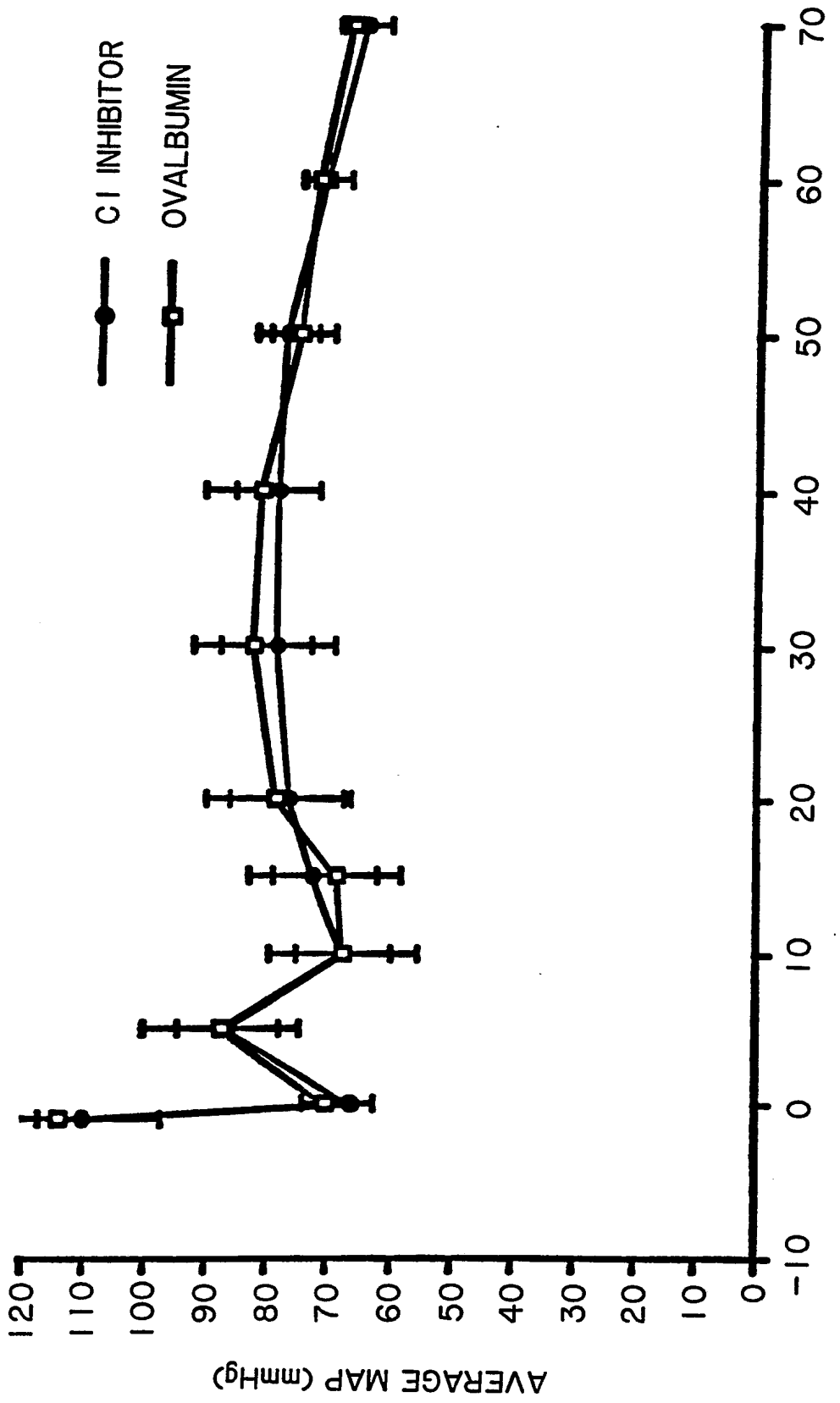
C1 INHIBITOR
OVALBUMIN
AVERAGE MAP (mmHg)
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
0
10
20
30
40
50
60
70
TIME (min)

FIG. 3

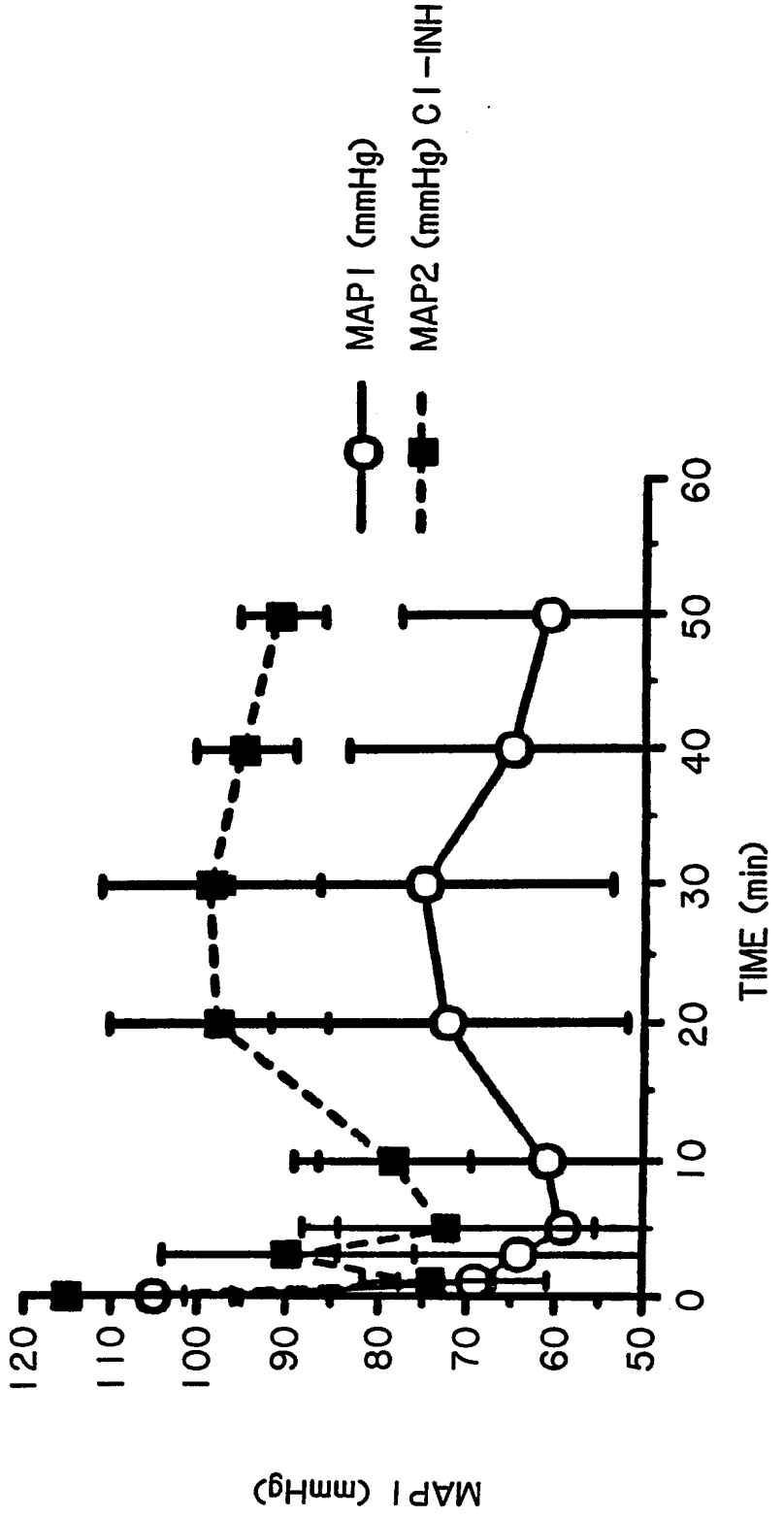
MAP1 (mmHg)
MAP2 (mmHg) C1-INH
120
110
100
90
80
70
60
50
0
10
20
30
40
50
60
TIME (min)
MAP1 (mmHg)

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/04452

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/64; C07K13/00; //C12N15/15

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	LANGENBECKS ARCHIV FÜR CHIRURGIE, CHIRURG. FORUM 85; ED. F. STELZNER; SPRINGER VERLAG BERLIN HEIDELBERG; 1985, pages 63 - 67; H. F. WELTER ET AL.: 'Versuche zur Therapie der Schocklunge mittels Superoxiddismutase (SOD) und C1-Inaktivator (C1-INA)' see the whole document ---	1,3-5
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 264, no. 6, 1989, BALTIMORE US pages 3066 - 3071; K. SKRIVER ET AL.: 'CpG Mutations in the Reactive Site of Human C1 Inhibitor' see abstract see page 3066, right column, paragraph 3 see page 3069, left column, paragraph 2 ---	8,9,14

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 SEPTEMBER 1992	11. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	THIELE U.H.-C.H.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	FEBS LETTERS. vol. 266, no. 1,2, June 1990, AMSTERDAM NL pages 13 - 16; K. S. AULAK ET AL.: 'Identification of a new P1 residue mutation (444Arg-Ser) in a dysfunctional C1 inhibitor protein contained in a type II hereditary angioedema plasma' see abstract	8,9,14
X	WO,A,9 106 650 (CETUS CORPORATION) 16 May 1991 cited in the application	9
A	see claims 1-6,8-13,22-27; figures 3,4	1-4
Y	BIOCHEM. J. vol. 258, no. 1, 1989, LONDON, UK pages 193 - 198; P. A. PEMBERTON ET AL.: 'The structural basis for neutrophil inactivation of c1 inhibitor' see page 1989, right column, paragraph 4 -paragraph 6; figure 4	8-11, 13-14
Y	PROTEIN ENGINEERING. vol. 1, no. 1, 1986, EYNSHAM, OXFORD, ENGLAND GB pages 29 - 35; S. JALLAT ET AL.: 'Altered specificities of genetically engineered alphal-antitrypsin variants' see page 34, left column, paragraph 1 -paragraph 2	8-11, 13-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/ 04452

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-7 are directed to a method of treatment of the human/ animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9204452 SA 61100

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 04/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9106650	16-05-91	AU-A- 6611290	31-05-91
		EP-A- 0497833	12-08-92